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Glycogen, Hyaluronate, and Some Other Polysaccharides Greatly Enhance the Formation of Exolipase by *Serratia marcescens*†

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Among 21 different polysaccharides tested, 5 greatly enhanced the spontaneous and cyclic AMP-induced formation of exolipase: glycogen, hyaluronate, laminarin, pectin B, and gum arabic. These polysaccharides have in common the tendency to form highly ordered networks because of the branching or helical arrangement, or both, of their molecules. None of the polysaccharides could be utilized by the cells as the sole carbon source. Strong lipid extraction of four different polysaccharides did not reduce their exolipase-enhancing efficacy. At a constant cell density the stimulation of exolipase formation by various concentrations of glycogen followed saturation kinetics, suggesting a limited number of "sites" for the glycogen to act. The active principle present in a solution of pectin was destroyed by degradation (β-elimination) of the polymer. Hyaluronate lost its exolipase-enhancing activity by exhaustive hydrolysis with hyaluronidase but was resistant to proteinase K. Exopolysaccharide, isolated from growth medium of *Serratia marcescens* SM-6, enhanced the exolipase formation as efficiently as hyaluronate. The results of this work are discussed mainly in terms of the "detachment hypothesis."

It was reported recently that the formation of extracellular lipase ("exolipase") by *Serratia marcescens* SM-6 was stimulated up to 100-fold when the standard growth medium was supplemented with glycogen or pectin B (26, 27). The present paper provides an initial experimental analysis and discussion of this new bacteriological phenomenon. Twenty-one different polysaccharides were screened for exolipase-enhancing activity to determine chemical or structural requirements for this effect. Various wild-type strains of *S. marcescens* were studied to see whether the polysaccharidic stimulation of exolipase formation is restricted to strain SM-6. Kinetic measurements of the effect of glycogen on the yield of exolipase activity were performed.

Most of the experiments described in this paper were performed with mutant strain W1270, a derivative of *S. marcescens* SM-6 (28). This mutant has the advantage that exolipase enhancement by polysaccharides can be studied within 30 min.

MATERIALS AND METHODS

Bacteria. The strains used mainly were *S. marcescens* SM-6 F'*lac*$^+$ (wild type) and double mutant W1270 (Cpd Cya), which lacks cyclic AMP (cAMP)-phosphodiesterase activity and requires exogenous cAMP for various cell functions, including the formation of exolipase (28). In addition, *S. marcescens* strains 862-57, 868-57, 4534-60, 6320-58, and 3607-60, kindly provided by B. Davis, Atlanta, Ga., were studied; these strains all differ with respect to their O- and H-antigens (Table 2). *Enterobacter liquefaciens* W1079 (syn. B1622) was obtained from E. T. Reese, Natick, Mass.

Media. Minimal medium M9 (M9 glc) contained 7 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 4 g of glucose, 0.25 g of $MgSO_4 \cdot 7H_2O$, and 0.02 g of $CaCl_2 \cdot 2H_2O$ per liter of demineralized water. Supplemented minimal medium (M9 glc-s) also contained 0.25% Casamino Acids (Difco). In some experiments glucose was replaced by glycerol.

Biochemicals. The polysaccharides used were of the highest grade of purity commercially available; they were purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Sigma (St. Louis, Mo.), and Pharmacia (Uppsala, Sweden). Pullulan was a gift from K. Wallenfels (Freiburg, Germany).

Induction of exolipase formation by cAMP. Minimal medium M9 glc-s was inoculated with mutant W1270 at an initial optical density at 580 nm (OD_{580}) of 0.5. After incubation on a gyratory shaker for 7 h at 30°C, the OD_{580} was usually 3.4. From this culture, 1 part was mixed with 1 part of prewarmed glucose-free M9 solution (diluted 1:5 in distilled water) and 1/25

† Dedicated to Max Delbrück with gratitude.

volume of a 0.1 M cAMP solution. After various times of shaking at 30°C, samples were quickly cooled in an ice bath and centrifuged (4 min at 8,000 × *g*). The cell-free supernatant fluids obtained were immediately assayed for exolipase activity.

Testing of the exolipase-enhancing ability of polysaccharides. Two different tests were performed. (i) The polysaccharides were added to the growth medium (10 ml of M9 glc) of *S. marcescens* SM-6 at a final concentration of 0.4%. After 22 to 24 h of incubation in a rolling apparatus at 30°C, the cell density (OD_{580}) was determined, the cells were removed by two successive centrifugations, and the supernatant fluids were assayed for exolipase, exoprotease, and exonuclease activity. One control tube contained plain M9 glc medium, and another one contained M9 medium in which glucose was replaced by 0.4% of the respective polysaccharide. All extracellular enzyme activities were normalized by dividing by the initial cell density (OD_{580}) of the original culture. (ii) Mutant W1270 was induced with 2 mM cAMP as described above, except that the 1:5 diluted glucose-free M9 solution contained the polysaccharide to be studied. All exolipase activities measured were corrected for background, i.e., the enzyme activity found in the absence of cAMP and of the particular polysaccharide. Boiling of the polysaccharide solutions before use (5 to 10 min) did not significantly alter their exolipase-stimulating effect except in the case of pectin B.

Isolation and chemical analysis of bacterial exopolysaccharide. *S. marcescens* SM-6 was grown in M9 sucrose (2%) medium on a gyratory shaker for 48 h at 30°C. After removal of the cells by centrifugation, crude exopolysaccharide was obtained by dialysis against tap water and freeze drying. The material was then incubated for 90 min with trypsin, pancreatic RNase, and DNase (10 μg of each ml^{-1}). After heat inactivation of the enzymes, dialysis, and freeze-drying, the product obtained was used as partially purified exopolysaccharide. A qualitative analysis of the polysaccharide was performed with a sample hydrolyzed with 1 N H_2SO_4 at 100°C for 24 h. The solution was neutralized with Amberlite IRA 410. Thin-layer chromatographic analysis was performed on cellulose plates (Merck 5716) with a mixture (4:6:3) of pyridine, *n*-butanol, and distilled water (11). For detection of hexoses, pentoses, hexosamines, and uronic acids the thin-layer chromatographic plates were developed with the appropriate reagent as described by Stahl (23).

Determination of protein and polysaccharide. Protein concentrations were estimated according to Lowry et al. (17), using bovine serum albumin as a standard. Polysaccharide concentrations were determined by weighing or by applying the phenol-sulfuric acid method (7), using dextran (molecular weight, 50,000) as a standard.

Assays of extracellular enzymes. Exolipase was assayed using *p*-nitrophenylpalmitate (Serva) as the substrate (M. Thöner, Diplom-Thesis, University of Bochum, Bochum, Germany, 1973). Ten milliliters of isopropanol containing 30 mg of *p*-nitrophenylpalmitate was mixed with 90 ml of 0.05 M Sörensen phosphate buffer, pH 8.0, containing 207 mg of sodium deoxycholate and 100 mg of gum arabic. A 2.4-ml amount of this freshly prepared substrate solution was prewarmed at 37°C and then mixed with 0.1 ml of cell-free supernatant fluid. After 15 min of incubation at 37°C, the OD_{410} was measured against an enzyme-free control. One enzyme unit is defined as 1 nmol of *p*-nitrophenol enzymatically released from the substrate ml^{-1} min^{-1}. Under the conditions described the extinction coefficient of *p*-nitrophenol is $\epsilon_{410} = 15,000$ cm^2 mg^{-1}. The exoprotease was assayed according to Charney and Tomarelli (5) with the following modifications. The enzyme substrate (azocasein, Serva) was dissolved in 0.1 M phosphate buffer (pH 7.6) at a concentration of 12.5 mg ml^{-1}. Two milliliters of this substrate solution was mixed with 1 ml of cell-free supernatant fluid and incubated at 30°C for 30 min. One enzyme unit is defined as $\Delta OD_{440} = 1.0$ ml^{-1} min^{-1}. The exonuclease was assayed with RNA as a substrate (8).

Enzymatic hydrolysis of hyaluronate. Potassium hyaluronate was dissolved in 0.3 M phosphate buffer, pH 5.3, and bovine testicular hyaluronidase (EC 3.2.1.35; Sigma) was added. After various times of incubation at 30°C, samples were taken and boiled for 3 min each, and the amount of undegraded hyaluronate was estimated by applying the procedure of Tolksdorf et al. (24). Details of the method are given in reference 2.

Heat-induced β-elimination of pectin (1). Pectin B was dissolved in 0.1 M sodium phosphate buffer, pH 6.8, at a concentration of 10 mg ml^{-1} and heated for various times at 95°C. Aliquots were taken to measure the decrease in viscosity (OSTWALD instrument, 0.6-mm capillary diameter, 22°C), the increase in absorbance at 235 nm, and the decay of the exolipase-enhancing capability.

Enzymatic de-esterification of pectin. Pectin B was dissolved in 0.05 N NaCl solution (5 mg ml^{-1}). After neutralization pectin methylesterase (EC 3.1.1.11, Sigma; 160 U/mg of protein) was added, and the solution (30°C) was kept at pH 7 by adding microliter amounts of 0.5 N NaOH until the de-esterification was completed.

RESULTS

Screening for exolipase-enhancing polysaccharides. Glycogen and pectin B are the only polysaccharides presently reported to enhance the formation of exolipase by *S. marcescens* SM-6 (26, 27). Here 19 additional polysaccharides and 2 polyvinyls were tested for the same effects. Three new exolipase-enhancing polysaccharides were found (Table 1). The other substances tested, a list of which follows, were inactive: arabic acid, sodium salt; amylopectin (?); amylose; carboxymethylcellulose; chondroitin sulfate, sodium salt; colominic acid, sodium salt; β-cyclodextrine; dextran (molecular weight, 60,000 to 90,000); dextran sulfate 500, sodium salt; Ficoll; inulin; mannan; polyethyleneglycol 6000 (?); polygalacturonic acid, sodium salt; polyvinylalcohol; polyvinylpyrrolidone; pullulan; starch. (The polymers were tested under the

TABLE 1. *Polysaccharides that enhance the formation of exolipase by S. marcescens SM-6*[a]

Polysaccharide	Chemical components[b]	Type of linkages[b]	Enzyme activities (U)		
			Exolipase	Exoprotease ($\times 10^3$)	Exonuclease ($\times 10^3$)
None			2.2 ± 1.0	5.0 ± 0.6	2.7 ± 0.6
Hyaluronate (Serva 25130)	GlcUA GlcNAc	β 1,3 β 1,4	13.1 ± 1.5	2.6 ± 0.1	2.0 ± 0.2
Laminarin (Roth 5971)	Glc (Man)	β 1,3 (β 1,6)	25.4 ± 6.7	4.2 ± 0.5	2.5 ± 0.1
Glycogen (Serva 23550)	Glc	α 1,4 (α 1,6)	16.7 ± 4.9	5.4 ± 0.3	1.9 ± 0.2
Pectin B (Roth 2-8909)	$GalUA_{met}$ (Gal, Ara, Rha)	α 1,4	16.6 ± 0.4	3.3 ± 0.1	2.6 ± 0.3
Gum arabic (Merck 4282)	Gal, GlcUA (Ara, Rha)	Various	12.8 ± 2.9	6.1 ± 0.2	2.1 ± 0.3

[a] The polysaccharides were tested according to method (i); see Materials and Methods.

[b] The data given in parentheses indicate less frequent components and linkages of the polysaccharides. Abbreviations: Glc, D-glucose; GlcUA, D-glucuronic acid; GlcNAc, *N*-acetylglucosamine; Man, D-mannitol; $GalUA_{met}$, partially methyl-esterified D-galacturonic acid; Ara, D-arabinose; Rha, L-rhamnose; Gal, D-galactose.

same conditions as those listed in Table 1. The question mark in parentheses denotes polysaccharides which showed weak enzyme-enhancing activity in some of the experiments.) The only structural feature which the exolipase-enhancing polysaccharides have in common is the tendency to form highly ordered networks because of the branching or helical arrangement, or both, of the molecules (for details see Discussion).

S. marcescens SM-6 was unable to utilize any of the exolipase-enhancing polysaccharides as a sole source of carbon and energy. Since it is well known that *S. marcescens* can readily grow at the expense of self-synthesized, intracellular glycogen, the fact that the bacteria did not grow on exogenous glycogen indicates that glycogen was not taken up by the cells. Experiments in search of partial enzymatic breakdown of exogenous glycogen by the growth medium of *S. marcescens* W1270 have failed so far. For this purpose cell-free M9 glc-s growth medium from stationary cultures was concentrated fivefold by Amicon filtration (BM 500) or evaporation under reduced pressure (35°C) and incubated with a glycogen solution for 60 min. The concentration of reducing sugars was found to be constant during this time. None of the 21 different polysaccharides tested affected the exonuclease activity of *S. marcescens* SM-6. Some polysaccharides, for example, pectin B, lowered the yield of exoprotease to about 50% (Tables 1 and 2).

In a growing culture of *S. marcescens* SM-6 supplemented with glycogen, extracellular lipase activity could be detected about 3 h earlier than in glycogen-free control experiments (Fig. 1). Similar results were obtained when hyaluronate and pectin B were used as supplements of the growth medium. Neither glycogen nor hyaluronate was able to increase the total amount of extracellular protein usually found in dialyzed growth medium of a stationary culture of *S. marcescens* SM-6 (about 45 $\mu g\ ml^{-1}$).

Screening for bacterial strains in which exolipase responds to polysaccharides. The exolipase activity of five wild-type strains of *S. marcescens* which differ at least serologically from strain SM-6 and from each other was studied after growing the cells in the presence and absence of glycogen and pectin B (Table 2). Although the absolute exolipase activities of these strains varied, all strains showed principally the same response to glycogen and pectin B as strain SM-6. This result indicated that the exolipase-enhancing effect is not restricted to bacteria with a very specific lipopolysaccharide structure (O-antigen pattern) on their cell surface. In support of this conclusion was the finding that the exolipase activity even of *E. liquefaciens* W1079 increased 3- to 10-fold when the growth medium of the cells (M9 glc-s) was supplemented with 0.4% glycogen.

Enhancement of cAMP-induced exolipase formation by selected polysaccharides. Recently we found that *S. marcescens* W1270, after growth in cAMP-free M9 glc-s medium, can be induced by 2 mM cAMP to produce exolipase within 10 to 30 min. This "short-term" system was then used to retest the polysaccharides listed in Table 1 and those found to be inactive (see above); all results were confirmed. Special problems of the polysaccharidic effect were studied as follows.

Kinetics. In short-term experiments with mutant W1270, glycogen (0.2%) was able to enhance the formation of exolipase five- to sixfold (Fig. 2, left). The cell density and exoprotease activity

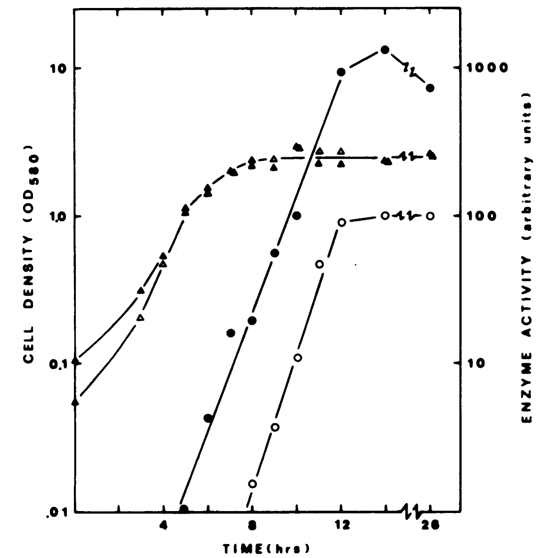

FIG. 1. *Effect of glycogen (0.6%) on cell growth and exolipase activity of S. marcescens SM-6 grown in M9 glucose medium for various times. The generation time of the cells in nonsupplemented medium was approximately 50 min. An exolipase activity of 100% corresponds to 63 nmol of p-nitrophenol ml^{-1} min^{-1}. Symbols: cell densities (▲, △); enzyme activities (●, ○); filled symbols indicate medium with glycogen and open symbols indicate medium without glycogen.*

did not change under these conditions (≤10%). The exolipase stimulation by glycogen was also studied as a function of its concentration: bacteria were first incubated solely with cAMP (0 to 30 min) and then were posttreated with different amounts of glycogen (30 to 35 min). The glycogen-induced increments of exolipase activity increased with the glycogen concentration according to saturation kinetics (Fig. 2, right). A double-reciprocal plot of the data (Fig. 2, insert) allowed us to calculate an apparent K_m of 0.22 mg of glycogen ml^{-1} and a V_{max} of 26 nmol of *p*-nitrophenol ml^{-1} min^{-1}. Assuming an average molecular weight of 10^5 for glycogen, the K_m calculated corresponds to about 2×10^{-6} M.

Dose-effect experiments were also performed with hyaluronate. An increase in the concentration of hyaluronate (0.2 to 4 mg/ml) caused an increased yield of exolipase, but the viscosity of highly concentrated solutions of this polysaccharide prevented accurate measurements.

Calcium ion effects. The yield of cAMP-induced exolipase activity of mutant W1270 increased when the $CaCl_2$ concentration of the growth medium was raised above normal (Fig. 3, curve A). Under the same conditions the ability of hyaluronate to stimulate the formation of exolipase decreased (Fig. 3, curve B). When $CaCl_2$ was replaced by Ca_2SO_4, the same effects were found; i.e., the anion seems to be rather unimportant. Corresponding experiments with glycogen and pectin B confirmed that polysaccharides stimulate the exolipase only at low Ca^{2+} concentrations.

Variation of the K^+ and Na^+ concentration in the growth medium of W1270 (20, 40, and 60 mM) did not alter the ability of hyaluronate to stimulate the formation of exolipase.

Use of *Serratia* exopolysaccharides. *S. marcescens* SM-6 produced extracellular polysaccharides ("exopolysaccharides") when grown in M9 glc medium. Usually, stationary cultures contained 40 to 60 μg of exopolysaccharide ml^{-1}. When exopolysaccharide was hydrolyzed by H_2SO_4 and afterwards analyzed by thin-layer chromatography the main constituents found were D-glucose and D-mannose. Minor components were L-rhamnose and L-fucose.

When added to mutant W1270 at the time of the cAMP treatment, partially purified exopolysaccharide was able to enhance the formation of exolipase at least as efficiently as hyaluronate (Fig. 4). Control experiments with a boiled mixture of trypsin, RNase, and DNase, but without exopolysaccharide, were negative. The *Serratia* exopolysaccharide also stimulated the spontaneous formation of exolipase of mutant W1270, but to less than 15% of the enzyme activity found after treating the cells with exopolysaccharide and cAMP. These results suggested (i) that the formation of exolipase in *S. marcescens* requires bacterial exopolysaccharides and (ii) that the polysaccharides of nonbacterial origin, listed in Table 1, enhance the formation of exolipase probably by mimicking and potentiating the action of the *Serratia* exopolysaccharides.

TABLE 2. *Effect of glycogen and pectin B on the formation of exolipase and exoprotease by six serologically different strains of S. marcescens*[a]

Strain no.	Serotype		Factor of enzyme stimulation			
			Exolipase		Exoprotease	
	O	H	Glycogen	Pectin	Glycogen	Pectin
SM-6[b]	?	?	7.3	8.4	1.2	0.46
862-57	6	3	2.5	5.1	1.1	0.54
868-57	2	1	3.5	8.4	1.3	0.32
4534-60	9	11	6.4	5.7	0.9	0.10
6320-58	12	9	2.8	10.2	0.8	0.34
3607-60	13	4	7.9	16.7	2.2	0.89

[a] The polysaccharides were tested according to method (i); see Materials and Methods. The M9 medium contained glycerol instead of the usual glucose.

[b] Strain SM-6 did not react with *Serratia* antisera O1 through O16 (D. J. Brenner, personal communication).

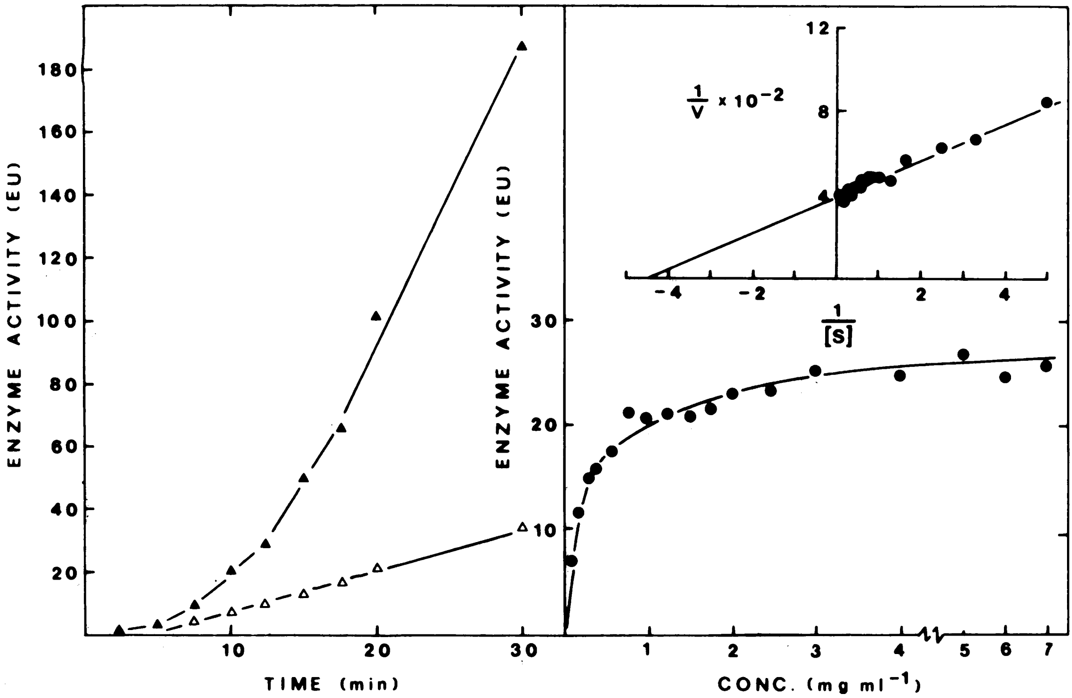

FIG. 2. *Effect of glycogen on the cAMP-induced formation of exolipase by S. marcescens W1270. (Left) Cells were incubated for various times with 2 mM cAMP in the presence and absence of 0.2% glycogen. (Right) cAMP-induced exolipase activity as a function of the concentration of glycogen. Experimental conditions: 10 ml of cell suspension (OD_{580} = 3.55) was mixed with 200 μl of 0.1 M cAMP and incubated for 30 min. Aliquots of 0.5 ml were then post incubated each with 0.5 ml of various glycogen solutions. The reaction was stopped by adding a few drops of toluene, which did not influence the lipase activity. (Insert) Double-reciprocal plot of the same data. The term V represents the increments of exolipase activity induced by glycogen treatment lasting 5 min.*

The polysaccharides themselves and not their contaminants seem to be the enhancing principle. Many preparations of natural polysaccharides contain, in addition to the polysaccharide chains, some proteins, metal ions, or even lipids. Experiments were performed to determine whether the exolipase-enhancing ability, for example, of pectin B and hyaluronate, is a feature of the polysaccharides themselves or of some of their non-polysaccharidic components.

Degradation and modification of pectin B. Heating of a neutral solution of pectin causes a nonhydrolytic splitting of the polymer (1). This so-called β-elimination was followed spectrophotometrically (235 nm) and viscosimetrically (Fig. 5). Figure 5 also shows that the exolipase-enhancing ability of pectin B decreased with increasing degree of pectin degradation.

The exolipase-enhancing ability of pectin B was also destroyed when pectin solutions were incubated with pectin methylesterase (0.26 U ml^{-1}) until the saponification was completed. This result agrees well with the observation mentioned above that polygalacturonate is unable to stimulate the formation of exolipase.

Enzymatic hydrolysis of hyaluronate. Solutions of potassium-hyaluronate were treated with hyaluronidase at 30°C. When high concentrations of the enzyme (750 U ml^{-1}) were applied for 4 to 9 h, the viscosity of the hyaluronate solution dropped to <2%, and its exolipase-enhancing ability decreased to ≤30%. This result again supported the assumption that the polysaccharides themselves are the "active principle." Heat-inactivated hyaluronidase itself was unable to influence the cAMP-induced formation of exolipase.

D-Glucuronic acid and *N*-acetyl-D-glucosamine are the smallest subunits of hyaluronate. Neither enhanced the cAMP-induced formation of exolipase by mutant W1270 when tested separately or after mixing in a 1:1 molar ratio (final concentration, 180 nM). On the contrary, *N*-acetyl-D-glucosamine abolished any formation of exolipase, even at a concentration as low as 3 mM.

Lipid extraction of polysaccharides. Lipid

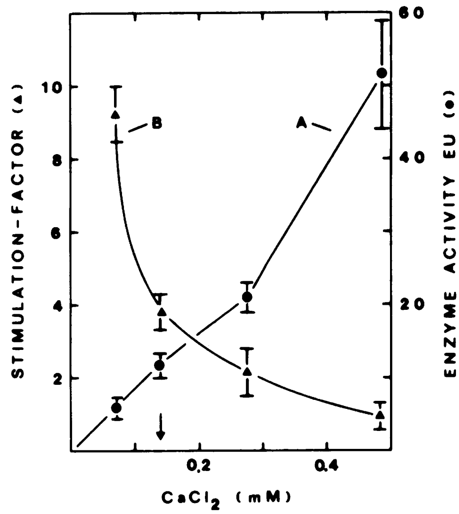

FIG. 3. *Effect on cAMP-induced exolipase formation of $CaCl_2$ concentration in the growth medium (M9 glc-s) of S. marcescens W1270. (Curve A) Exolipase activities, each induced by a 30-min treatment of the bacteria with 2 mM cAMP. All values represent the average of two independent experiments. In this particular experiment the substrate solution for the exolipase was prepared with 0.05 M Tris-hydrochloride buffer, pH 8.0, instead of the usual phosphate buffer. (Curve B) Factor by which the exolipase activity became increased when 0.4% hyaluronate was present in the cell suspension during the cAMP treatment. The arrow indicates the concentration of $CaCl_2$ in standard M9 medium. EU, Enzyme units.*

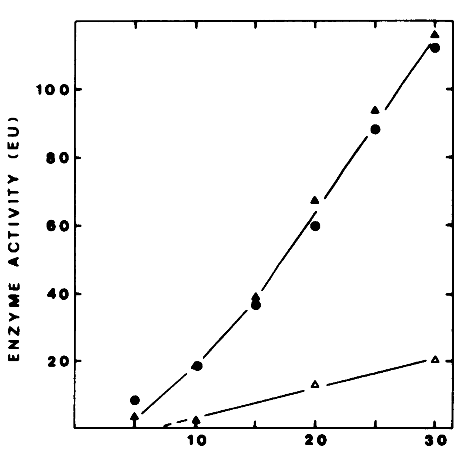

FIG. 4. *Effects of hyaluronate and exopolysaccharide derived from S. marcescens SM-6 on cAMP-induced exolipase formation by mutant W1270. The cells were incubated for various times with 2 mM cAMP in the presence and absence of 0.3% of the respective polysaccharide. Symbols: potassium hyaluronate (▲); bacterial exopolysaccharide (●); no supplement (△). EU, Enzyme units.*

extraction of polysaccharides was performed with glycogen, pectin B, laminarin, and gum arabic. Samples of the particular polysaccharide were subjected to either an ether-acetic acid (12) or a chloroform-methanol (Soxhlet) extraction (29). Neither of these lipid-extracting procedures affected the ability of the polysaccharides to enhance the formation of exolipase in mutant W1270. Furthermore, glycogen did not lose its exolipase-enhancing ability after prolonged incubation of the polysaccharide with partially purified *Serratia* exolipase (310 enzyme units for 60 min). These results eliminated the possibility that the exolipase-enhancing effect, caused by untreated polysaccharide, was simply due to substrate induction of the exolipase by some unknown lipoid impurity in the preparation used.

Treatment of polysaccharides with proteinase K. The preparation of potassium-hyaluronate used contained approximately 1.8% protein. Aqueous solutions of hyaluronate were treated with 210 μg of proteinase K ml^{-1} for 9 h and then boiled for 3 min. This enzyme treatment caused a reduction of the viscosity of the solutions to about 50% and doubled the ability of hyaluronate to enhance the cAMP-induced formation of exolipase. Thus, some of the protein present in the untreated hyaluronate prepara-

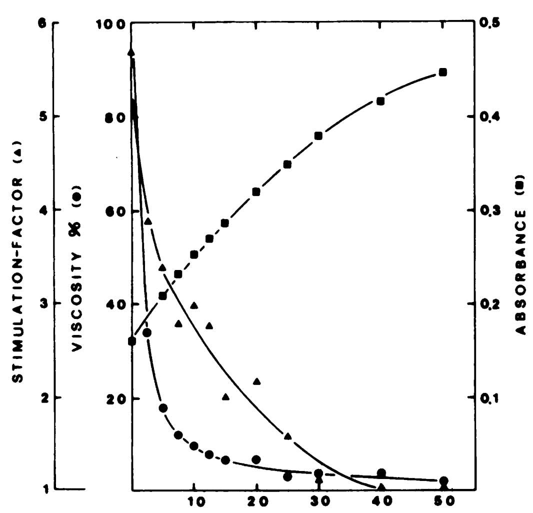

FIG. 5. *Viscosity, light absorbance (235 nm), and exolipase-enhancing capability of a 1% solution of pectin B after various times of heating at 95°C. For optical measurements the pectin solution was diluted 1:30. Conditions of the biological test: S. marcescens W1270, 2 mM cAMP, 0.5% pectin B, 40-min incubation. The exolipase activity of mutant W1270, treated with cAMP in the absence of pectin B, was 23 enzyme units.*

tion might have hindered the polysaccharide molecules from assuming that conformation which permits the most efficient stimulation of the formation of exolipase. Treatment of glycogen with proteinase K (150 μg ml^{-1} for 9 h) did not change its exolipase-enhancing ability.

Metal ions. Under chemically defined conditions (e.g., Tris-hydrochloride buffer), the cAMP-induced formation of exolipase by mutant W1270 requires potassium and calcium ions; the optimum concentrations found were 50 and 1 mM, respectively (I. Stumm and U. K. Winkler, unpublished data). Thus, it was conceivable that the exolipase-enhancing polysaccharides might function as suppliers of these required ions. For this reason the content of K^+, Na^+, and Ca^{2+} was determined in four different polysaccharide samples. The result of this experiment (Table 3) showed, however, that the above assumption was unlikely to be true. For example, hyaluronate, when used in biological experiments at a concentration of 0.4%, supplied potassium and calcium ions only at a concentration of 4.5 and 0.03 mM, respectively, whereas the corresponding optimal concentrations are at least 10 times higher.

DISCUSSION

This paper deals with the formation of exolipase by *S. marcescens* cells and its selective stimulation by a variety of exogenous polysaccharides.

Which polysaccharides are able to enhance the formation of exolipase? Among 21 different polysaccharides studied, only five enhanced the formation of exolipase significantly (see Table 1 and Results). These five differ in many respects but have in common a high degree of structural order enabling interstrand molecular interactions (16, 21): glycogen and gum arabic molecules are highly branched; the latter is said to be helically configurated also (10, 14). Hyaluronate, pectin, and laminarin molecules are either not or only rarely branched, but occur frequently as (sometimes "kinked") helices (6, 9, 19, 20). Therefore, it is now proposed that helical conformation or high branching, or both, are structural prerequisites for polysaccharides to enable them to enhance the formation of exolipase by *S. marcescens*. This hypothesis gained support from results of some recent experiments (K. E. Jäger, M. Portales, and U. K. Winkler, manuscript in preparation). (i) Glycogen lost its exolipase-enhancing ability when it was debranched with sweet potato β-amylase. Here, it is useful to recall that amylopectin, which is some kind of a "low-branched glycogen" (14), showed only very little exolipase-enhancing activity compared to that of glycogen. (ii) Hyaluronate, having been changed in molecular conformation by methylesterification of all its carboxyl groups, was unable to enhance the formation of exolipase.

TABLE 3. *Concentration of Na^+, K^+, and Ca^{2+} in exolipase-enhancing polysaccharides*[a]

Polysaccharide	Concn (mg g^{-1})		
	Na^+	K^+	Ca^{2+}
Hyaluronate (potassium salt)	0.43	43.8	0.29
Glycogen	2.37	1.75	3.12
Pectin B	0.87	3.75	3.12
Laminarin	0.59	0.63	0.13

[a] The polysaccharides were dissolved in double-distilled water. The concentration of the three metal ions was determined, using 0.08% solutions of the polysaccharides, with a Perkin-Elmer atomic absorption spectrophotometer, model 403 (temperature, ca. 2,400°C; 4:6 mixture of acetylene and air).

Enhancement of exolipase formation by polysaccharides: hypotheses on the mechanism. The cellular yield of exolipase increased according to saturation kinetics when the enzyme formation was studied as a function of the glycogen concentration (Fig. 2, insert). This saturation kinetics might indicate a limited number of "sites" for the glycogen molecules to act. In the following we propose the "detachment hypothesis." (i) The hypothetical "sites" are located on or near the cell surface and are places for temporary, noncovalent binding of newly synthesized exolipase molecules. (ii) The exolipase-enhancing polysaccharides detach the cell-bound exolipase through competition for the site (i.e., steric exclusion) or by changing the conformation of the exolipase. This concept was first developed to explain interactions between heparin and lipoprotein lipase (4, 15, 18). (iii) Bacteria which are incubated in the presence of an exolipase-enhancing polysaccharide for longer periods of time (Fig. 1) continuously refill the "exolipase gaps" at the cell surface, thereby producing excessive amounts of exolipase.

Two alternatives to the detachment hypothesis which seem less likely to be true are the following. (i) In the enzyme protection hypothesis the exogenous polysaccharides might protect nascent chains of exolipase from proteolysis at a vulnerable stage of the secretion process. This hypothesis was developed when protoplasts from gram-positive bacteria were studied (3). (ii) In the membrane pore hypothesis the exogenous polysaccharides might complex with polysaccharide chains at the cell surface (21) and reduce the fluidity of the outer membrane (22), thereby helping the formation of relatively stable pore-like structures (25) for the transmembrane export of exolipase molecules.

The question of why the enzyme-enhancing polysaccharides acted on exolipase but not on exoprotease and exonuclease (Table 1) remains. One answer could be that exoenzymes which are glycoproteins might preferentially bind to (polysaccharidic) cell surface sites (13) and respond to enzyme-enhancing polysaccharides. The exolipase produced by *S. marcescens* strain HY contains approximately 50% (wt/wt) firmly bound carbohydrate (J. Sossinka, personal communication).

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